Impaired vasoreactivity despite an increase in plasma nitrite in patients with abdominal aortic aneurysms

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Objective: **This investigation was designed to determine whether differences in vasoreactivity occur in patients with abdominal aortic aneurysms (AAAs) as compared with patients with peripheral arterial occlusive disease (PAOD) or individuals (controls) without known vascular disease.**

Methods: **Brachial artery vasoreactivity was assessed in a blinded fashion, after endothelium-dependent (ED) and endothelium-independent (EI) flow-mediated vasodilation, in age-matched, male patients with AAAs (n = 11) or PAOD (n = 9) or in controls (n = 10). There were no significant differences in prestudy systolic or diastolic blood pressure, body mass index, or antilipidemic medications among the groups studied. Exclusion criteria included diabetes and tobacco use within 3 months. Quantitative ultrasound scan measurements of brachial artery diameters were performed at rest and after either forearm ischemia (ED) or administration of 0.4 mg sublingual nitroglycerin (EI). Plasma nitric oxide ($NO_X = NO_2 + NO_3$) was measured with the Saville assay. Asymmetric dimethylarginine, an endogenous inhibitor of NO_X synthase, was measured with liquid chromatography.**

Results: **Initial brachial artery diameters were not significantly different among the groups studied (4.85 ± 0.18 mm for AAA group, 4.82 ± 0.17 mm for PAOD group, 4.68 ± 0.20 mm for controls). ED and EI vasodilation was significantly less ($P = .02$ and .03, respectively) in the AAA group (–1.71 ± 1.52 and 8.33 ± 1.13, respectively) when compared with the controls (2.96 ± 1.04 and 13.88 ± 2.16, respectively). However, plasma NO_X was significantly increased ($P = .01$) in the AAA group (7.86 ± 0.85 μmol/L) as compared with both controls (5.13 ± 0.63 μmol/L) and PAOD (4.85 ± 0.46 μmol/L). Asymmetric dimethylarginine levels were decreased in the AAA group (0.34 ± 0.05 μmol/L) as compared with the PAOD group (0.46 ± 0.09 μmol/L). No correlation existed between aneurysm size and ED or EI vasodilation or plasma NO_X.**

Conclusion: **This study is the first to document a divergence between ED and EI vasoreactivity and systemic NO metabolites in patients with AAAs. It is speculated that a dysfunctional vessel wall response, rather than a lack of NO, may be important in the pathogenesis of AAAs. (J Vasc Surg 2002;35:363-7.)**

Patients with abdominal aortic aneurysms (AAAs) are known to be at risk for the development of femoral and popliteal artery aneurysms, which suggests the existence of a generalized disease process.[1-3] Furthermore, insight into aneurysmal disease may be forthcoming from observations of vein grafts placed in patients with and without arterial aneurysms. For example, autogenous bypass grafts performed for popliteal aneurysmal disease tend to dilate over time as compared with vein grafts for occlusive disease that do not.[4] In this regard, vein graft aneurysms arose in 10 of 24 patients (42%) with arterial aneurysms in contrast to four of 221 patients (0.02%) who underwent treatment for occlusive disease ($P < .001$).[5] It is uncertain whether such changes are structural in character or are the result of differences in vasoactive substances.

This investigation was designed to determine whether differences existed in brachial artery vascular reactivity in patients with AAAs in comparison with those with occlusive vascular disease and healthy control subjects and, if differences existed, whether they reflected differences in known mediators of vasomotion.

METHODS

Patient selection. Eleven patients with AAAs, nine patients with peripheral arterial occlusive disease (PAOD), and 10 individuals without vascular disease were studied. The subjects were all male, nondiabetic, nonsmokers and ranged in age from 44 to 85 years. Cardiovascular risk fac-

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Characteristics of experimental groups investigated

	AAA (n = 11)	*PAOD (n = 9)*	*Control (n = 10)*
Age (years)	69.2 ± 2.9	67.9 ± 3.0	70.5 ± 3.1
Current tobacco use	0 (0)	0 (0)	0 (0)
History of tobacco use	10 (91%)	7 (78%)	5 (56%)
Body mass index	29.2 ± 1.2	26.2 ± 0.9	25.6 ± 1.1
Systolic BP (mm Hg)	132 ± 4	144 ± 4	129 ± 8
Diastolic BP (mm Hg)	74 ± 2	77 ± 2	74 ± 7
Myocardial infarction	5 (45%)	2 (22%)	0 (0)
Congestive heart failure	3 (27%)	1 (11%)	0 (0)
Angioplasty	3 (27%)	1 (11%)	0 (0)
CABG	4 (36%)	1 (11%)	0 (0)
CVA/TIA	2 (18%)	3 (33%)	0 (0)
Antilipidemic medications	6 (55%)	3 (33%)	1 (17%)
Antihypertensive medications	11 (100%)	6 (67%)	0 (0)

AAA, Abdominal aortic aneurysm; *PAOD,* peripheral arterial occlusive disease; *BP,* blood pressure; *CABG,* coronary artery bypass graft; *CVA,* cerebral vascular accident; *TIA,* transient ischemic attack.

tors, medical and surgical history, current medications, and physical parameters, including body mass index and blood pressure, were assessed. The study was approved by the University of Michigan Institutional Review Board for Human Subjects #1993-446.

Data collection. The patients underwent ultrasonic evaluation of brachial artery reactivity with a well-established methodology[6] in the University of Michigan Clinical Research Center. Brachial artery diameters at baseline, after reactive hyperemia, and after sublingual nitroglycerin administration exhibited no significant interobserver or intraobserver variation (data not shown).

Brachial artery images were obtained with a Hewlett-Packard 10-MHz linear array transducer and an Image Point ultrasound scan system (Hewlett Packard, Andover, Mass). Imaging was performed with the patient resting supine quietly for at least 10 minutes in a light-controlled and temperature-controlled room, with electrocardiographic leads placed for image capture synchronization. After baseline measurements, a blood pressure cuff was inflated to 200 mm Hg on the proximal right forearm for 4 minutes. The brachial artery was imaged 1 minute after the release of the cuff. This endothelium-dependent (ED) response was followed by a return to baseline during a period of 15 minutes. An endothelial-independent (EI) response then was evaluated with 0.4 mg of nitroglycerin administered sublingually. The brachial artery images were obtained 3 minutes after nitroglycerin administration. After a footswitch trigger event, six sequential frames were acquired (at a rate of 30 frames per second) through a DT3152 video capture board (Data Translation Inc, Marlboro, Mass) attached to a computer. This sequence was repeated four times for a total of 24 images. The time interval between each trigger event was left to operator discretion. The endpoint of measurement was the percent change in vessel diameter in response to reactive hyperemia or nitroglycerine administration.

The images were analyzed by the measurement of the average vessel diameter for each image set, defined as the distance between the intima-media interface on opposite sides of the vessel. The flow mediated diameter ratio (FMDR) then was calculated with the ratio of the change in vessel diameter to the baseline diameter as defined by the following equation, where FMD_A and FMD_B represent flow-mediated vessel diameters after intervention and at baseline, respectively: $FMDR = [(FMD_A - FMD_B)/FMD_B] \times 100$.

Serum collection. At the conclusion of the study, 10 mL of whole blood was collected from the patient's contralateral antecubital vein into vacutainers that contained either ethylenediamine tetraacetic acid or heparin. Immediately after collection, the samples were placed on ice. The samples were centrifuged within 1 hour of collection at $1800 \times g$ for 10 minutes at 4°C, and the plasma was stored at –70°C until it was analyzed at a later time.

Measurement of nitric oxide. A standard colorimetric assay (Cayman Chemical, Ann Arbor, Mich) was used to measure plasma nitric oxide (NO_x; nitrate and nitrite, oxidative metabolites of endogenous NO). Briefly, nitrate was converted to nitrite with nitrate reductase, followed by the addition of Griess reagents, which convert nitrite to a deep purple azo compound that is then measured spectrophotometrically and compared with a known standard curve. Total plasma NO_x was expressed in μmol/L.

Measurement of asymmetric dimethylarginine. Asymmetric dimethylarginine (ADMA) was separated and quantified with reverse-phase liquid chromatography (Waters Xterra MS C18, 4.6 mm × 250 mm, 3.5 μm with a 3.9 mm × 20 mm guard column at 36°C; Milford, Mass) after precolumn fluorescent derivation (Waters AccQ•Fluor). A two-pump gradient system (Waters HPLC, Model 510 Solvent Delivery System) delivered 87% 10 mmol/L 4-methylmorphyline and 13% methanol at 1 mL/min for 65 minutes. Standards, blanks, and samples (10 μL) were automatically injected (Model 712 Waters Intelligent Sample Processor), and fluorescent peak height and area were evaluated at an excitation of 250 nm and an emission of 395 nm (Waters 474 Scanning Fluorescence Detector),

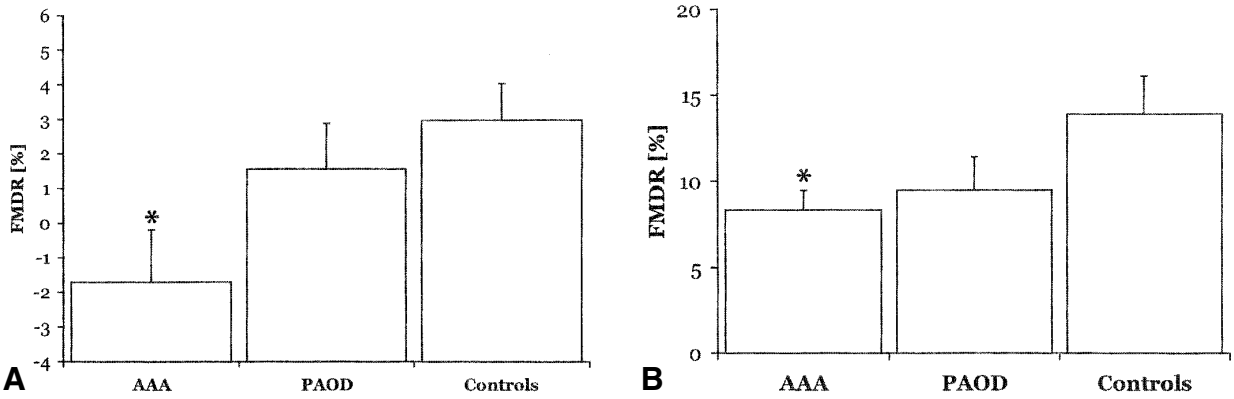

Fig 1. A, After reactive hyperemia, flow mediated diameter ratio *(FMDR)* of patients with abdominal aortic aneurysms *(AAAs)* was significantly less ($P = .02$) than that of healthy controls. FMDR of patients with peripheral arterial occlusive disease *(PAOD)* was less than that of healthy controls, but difference was not statistically significant ($P = .41$). **B,** After administration of nitroglycerine, FMDR of patients with AAAs was significantly less ($P = .03$) than that of healthy controls. FMDR of patients with PAOD was less than that of healthy controls, but difference was not statistically significant ($P = .15$).

with standard curves from 10.0 μmol/L to 0.1 μmol/L original concentrations. Only AAA (n = 5) and PAOD (n = 6) heparinized samples were obtained for evaluation of ADMA levels.

Statistical analysis. The data in this study are reported as mean ± standard error of the mean for numerical results and as group percentage for categorical results. FMDR, NO_x, and ADMA were calculated as mean ± standard error of the mean for each group. Student *t* test then was applied to the numerical results, and χ^2 analysis was applied to the categorical results with a StatView software package (SAS Inc, Cary, NC).

RESULTS

The study groups were well-matched by age, gender, body mass index, systolic blood pressure, diastolic blood pressure, comorbidities, and cardiovascular risk factors (Table). Initial brachial artery diameters were nearly identical in all three groups (4.85 ± 0.18 mm for AAA, 4.82 ± 0.17 mm for PAOD, 4.68 ± 0.20 mm for control; $P = .77$). No significant correlations were found between either ED or EI vasoreactivity, NO_x and age, body mass index, or diastolic or systolic blood pressure ($P > .05$; data not shown). No significant variations were observed between initial brachial artery diameter measurements and measurements after return to baseline after the 15-minute rest period ($P > .05$ for all groups).

After reactive hyperemia, the FMDR of the AAA group (–1.71 ± 1.52) was significantly lower ($P = .02$) than that of the healthy controls (2.96 ± 1.04), and the FMDR of the PAOD group (1.55 ± 1.31) ranged between that of the control and the AAA groups and was not significantly different from the AAA group or the healthy controls (Fig 1*A*). After nitroglycerin administration, the FMDR of the AAA group (8.33 ± 1.13) was significantly lower ($P = .03$) than that of the healthy controls (13.88 ± 2.16). The FMDR of the PAOD group (9.47 ± 1.90) ranged between that of the control and the AAA groups and was not significantly different from the AAA or control groups (Fig 1*B*).

Plasma NO_x levels (Fig 2) in the AAA group (7.86 ± 0.85 μmol/L) were significantly increased ($P = .04$) as compared with the PAOD group (4.85 ± 0.46 μmol/L) and the control group (5.13 ± 0.63 μmol/L). No statistically significant differences between the PAOD and the control groups were observed. Plasma ADMA levels (Fig 3) in the AAA group (0.34 ± 0.05 μmol/L) were decreased 26% as compared with the PAOD group (0.46 ± 0.09 μmol/L), although the difference did not reach significance ($P = .30$).

DISCUSSION

In this investigation, decreased vascular reactivity in response to a normal stimulus occurred in patients with AAAs as compared with healthy control patients. Despite diminished vessel reactivity, systemic NO metabolites were significantly higher in patients with AAAs. Similar findings have been found in rabbits fed high-cholesterol diets.[7] This discrepancy suggests that blood vessels in patients with aneurysms do not respond in a normal manner to local NO, despite being of comparable initial size to vessels in healthy patients. Persistence of this effect with nitroglycerine administration suggests that this may be a result of a defective vessel wall, not a deficiency in endogenous nitrates. The apparent decrease in ADMA, an endogenous inhibitor of nitric oxide synthase (NOS), in patients with aneurysms supports the tenet that the dysregulation of NO metabolism in the vessel wall occurs as a systemic alteration.

Vessel wall relaxation is known to be regulated by endothelium-derived NO by way of a cyclic guanosine monophosphate–mediated mechanism.[8-10] N^G-nitro-L-arginine methyl ester, a NOS antagonist, impairs ED vasodilation of the brachial artery of healthy volunteers,

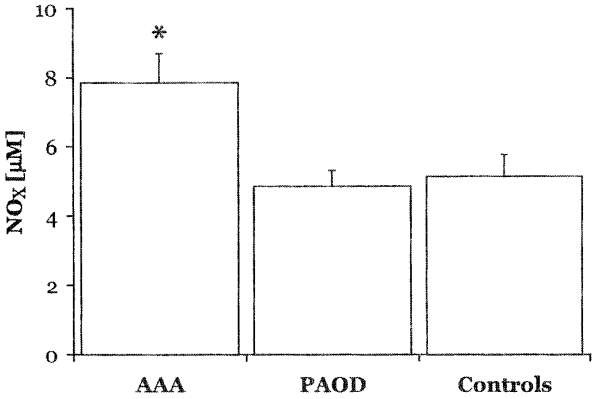

Fig 2. Serum nitric oxide *(NO_x)* levels in patients with an abdominal aortic aneurysm (AAA) were significantly higher than those in healthy controls ($P = .01$) and in patients with peripheral arterial occlusive disease (*PAOD;* $P = .01$). There was no significant difference in serum NO_x levels between patients with PAOD and healthy controls ($P = .74$).

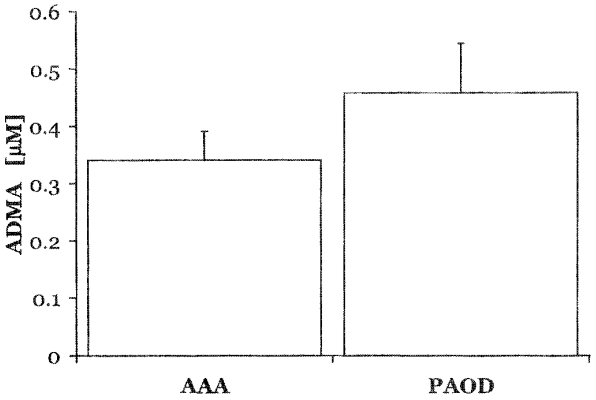

Fig 3. Serum asymmetric dimethylarginine *(ADMA)* levels in patients with an abdominal aortic aneurysm (AAA) were decreased 26% as compared with those of patients with peripheral arterial occlusive disease *(PAOD)*, although this decrease was not stastitically significant ($P = .30$).

yet it has no effect on EI vasodilation, highlighting the central role of NO in ED vasodilation.[11]

There is growing evidence that both aneurysmal and occlusive vascular diseases are characterized by systemic alterations in vascular reactivity.[12,13] It has been previously asserted that a reduction in iliac artery vascular reactivity in patients with AAAs is a result of endothelial cell dysfunction caused by atherosclerotic changes in the vessel wall.[14] A major limitation of the former study was the use of the common iliac artery to investigate systemic vascular reactivity. The common iliac artery is commonly aneurysmal in patients with AAAs and therefore is not representative of healthy physiologic vascular responses in these individuals. In addition, the former study erroneously reported a nonsignificant decrease in EI vascular reactivity to indicate that no impairment existed, but their study was too underpowered ($\beta = .19$) to come to such a conclusion. The raw data of this prior study suggest that both ED and EI vascular reactivity are impaired in patients with AAAs. The fact that discrepancies in vascular reactivity and NO levels were detected in the brachial artery of patients with AAA and PAOD in this investigation suggests that these changes are not the result of atherosclerotic disease alone but are likely to be multifactorial.

Possible mechanisms for elevated plasma NO_x in patients with AAAs, in view of reduced intimal and medial reactivity, include a number of mechanisms. First, infiltration of inflammatory macrophages in the diseased vessel wall may play a role. It is generally accepted that aneurysms are associated with an inflammation within the media of the vessel.[15-18] As an aneurysm progresses, the increase in leukocytes, predominantly macrophages, in this prooxidative environment may result in significantly elevated NO serum levels, despite a less responsive media. A second possibility would be a defect in the negative feedback regulation of NO synthesis. Local metabolites in regions of mild ischemia may induce increased vessel reactivity through the formation of NO by vascular smooth muscle cells, but an inability of these cells to respond to NO in a paracrine fashion would prevent down-regulation of NO synthesis and lead to elevated serum levels of NO. Third, either an impairment in the incorporation of NO into a more potent parent compound or a decreased half-life of NO has been suggested to explain these paradoxical findings.[7] Deficiency of tetrahydrobiopterin, a cofactor of NOS, is another possible explanation for the altered metabolism of NO in AAAs.[19] Another cofactor of NOS, calcium-calmodulin complexes, may also be involved in this process. Defective calcium handling by both endothelial cells and smooth muscle cells would result in elevated cytoplasmic calcium, increasing NOS activity in endothelial cells and impairing vasorelaxation in vascular smooth muscle cells. Finally, NO metabolism may also produce a metabolite, such as S-nitroso-homocysteine, that is damaging to the vasculature.[20]

Regardless of the cause of the elevated NO metabolites, diminished vascular reactivity in patients with aneurysms in response to systemically administered nitrates and the paradoxical vasoconstriction in response to reactive hyperemia suggest a desensitization of the systemic vasculature to normal vasodilatory stimuli. These stimuli, when chronic, may lead to remodeling of the arterial wall, limiting the vessel responsiveness and contributing to the pathogenesis of aneurysm formation. This tenet is further supported by the apparent decreases in levels of ADMA in patients with aneurysms that would ordinarily be expected to improve vascular reactivity. An impairment of a response to NO by a dysfunctional vessel wall could explain these findings.

The findings of this investigation appear important, but certain study limitations must be considered in interpreting the data. First, the patient sample was relatively

small and potentially heterogeneous, especially in regards to the incidence rate of myocardial infarction, the history of tobacco use, and the need for antihypertensive medications. In addition, alternative methods for evaluating NO activity, including measurements of serum platelet cyclic guanosine monophosphate and free NO by chemiluminescence, were not pursued in this study. Furthermore, NO metabolites were not differentiated, although recent studies have suggested that variable bioactivity exists among these metabolites.[21,22] Finally, there are several additional soluble mediators of vascular function that may play a role in the pathophysiology of aneurysmal disease not assessed in this study.

A relevant issue relates to the effect of operative repair of the AAA or occlusive disease on systemic vascular reactivity. Postoperative studies might determine whether the observed differences among the groups studied represent a constitutional predisposition towards diminished reactivity in patients with aneurysms or whether it is simply a result of the local aortic disease.

Despite these limitations, this investigation revealed differences in the physiologic response of blood vessels in patients with AAAs as compared with those with occlusive disease and matched controls, despite increased circulatory serum NO metabolite levels. This paradigm sets the stage for future investigations aimed at determining the pathophysiologic role of NO in aneurysm initiation and propagation.

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REFERENCES

1. Diwan A, Sarkar R, Stanley JC, Zelenock GB, Wakefield TW. Incidence of femoral and popliteal artery aneurysms in patients with abdominal aortic aneurysms. J Vasc Surg 2000;31:863-9.
2. Graham LM, Zelenock GB, Whitehouse WM Jr, Erlandson EE, Dent TL, Lindenauer SM, et al. Clinical significance of arteriosclerotic femoral artery aneurysms. Arch Surg 1980;115:502-7.
3. Whitehouse WM Jr, Wakefield TW, Graham LM, Kazmers A, Zelenock GB, Cronenwett JL, et al. Limb-threatening potential of arteriosclerotic popliteal artery aneurysms. Surgery 1983;93:694-9.
4. Upchurch GR Jr, Gerhard-Herman MD, Sebastian MW, Belkin M, Conte MS, Donaldson MC, et al. Improved graft patency and altered remodeling in infrainguinal vein graft reconstruction for aneurysmal versus occlusive disease. J Vasc Surg 1999;29:1022-30.
5. Loftus IM, McCarthy MJ, Lloyd A, Naylor AR, Bell PR, Thompson MM. Prevalence of true vein graft aneurysms: implications for aneurysm pathogenesis. J Vasc Surg 1999;29:403-8.
6. Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet 1992;340:1111-5.
7. Minor RL Jr, Myers PR, Guerra R Jr, Bates JN, Harrison DG. Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta. J Clin Invest 1990;86:2109-16.
8. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993;329:2002-12.
9. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980;288:373-6.
10. Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 1988;333:664-6.
11. Lieberman EH, Gerhard MD, Uehata A, Selwyn AP, Ganz P, Yeung AC, et al. Flow-induced vasodilation of the human brachial artery is impaired in patients 40 years of age with coronary artery disease. Am J Cardiol 1996;78:1210-4.
12. Harris LM, Faggioli GL, Shah R, Koerner N, Lillis L, Dandona P, et al. Vascular reactivity in patients with peripheral vascular disease. Am J Cardiol 1995;76:207-12.
13. Sonesson B, Hansen F, Lanne T. Abdominal aortic aneurysm: a general defect in the vasculature with focal manifestations in the abdominal aorta? J Vasc Surg 1997;26:247-54.
14. Komori K, Mawatari K, Itoh H, Sugimachi K. Impaired vasodilation of peripheral arteries in response to acetylcholine in human beings with abdominal aortic aneurysm. J Vasc Surg 1994;20:803-7.
15. Newman KM, Jean-Claude J, Li H, Ramey WG, Tilson MD. Cytokines that activate proteolysis are increased in abdominal aortic aneurysms. Circulation 1994;90:II224-7.
16. Brophy CM, Reilly JM, Smith GJ, Tilson MD. The role of inflammation in nonspecific abdominal aortic aneurysm disease. Ann Vasc Surg 1991;5:229-33.
17. Rose AG, Dent DM. Inflammatory variant of abdominal atherosclerotic aneurysm. Arch Pathol Lab Med 1981;105:409-13.
18. Walton LJ, Franklin IJ, Bayston T, Brown LC, Greenhalgh RM, Taylor GW, et al. Inhibition of prostaglandin E2 synthesis in abdominal aortic aneurysms: implications for smooth muscle cell viability, inflammatory processes, and the expansion of abdominal aortic aneurysms. Circulation 1999;100:48-54.
19. Stroes E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, et al. Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. J Clin Invest 1997;99:41-6.
20. Stamler JS, Osborne JA, Jaraki O, Rabbani LE, Mullins M, Singel D, et al. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J Clin Invest 1993;91:308-18.
21. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, et al. Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. Proc Natl Acad Sci U S A 1992;89:7674-7.
22. Gladwin MT, Ognibene FP, Pannell LK, Nichols JS, Pease-Fye ME, Shelhamer JH, et al. Relative role of heme nitrosylation and β-cysteine 93 nitrosation in the transport and metabolism of nitric oxide by hemoglobin in the human circulation. Proc Natl Acad Sci U S A 2000;97:9943-8.

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